Effect of DHA on the quality of In vitro produced bovine embryos

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Theriogenology

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--Manuscript Draft--

Manuscript Number:	THERIO-D-22-00142R2
Article Type:	Original Research Article
Keywords:	cattle; IVP; n-3 PUFA; Cryopreservation; SOX2
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Effect of DHA on the quality of *In vitro* Produced Bovine Embryos

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1. Introduction

The ability of oocytes to develop *in vitro* into viable embryos after fertilisation is mainly acquired during final oocyte growth and meiotic maturation [1]. However, while the intrinsic quality of the oocyte is the main factor affecting the embryo developmental rate, the main factor affecting the quality of blastocysts is the post-fertilisation culture condition [2]. Freezing impairs the developmental potential of bovine embryos especially ICM cells [3]. In addition, membrane damage and DNA fragmentation induced by cryopreservation, reduce embryonic competence [4]. Thus, post-thaw establishment of ICM and TE and expression of the markers for their development competence could be relevant indicators of the quality of bovine embryos prior to embryo transfer.

The lipid composition of oocytes and embryos affects their development and their cryotolerance [5]. Lipid supplementations was used to increase embryo developmental rates in cattle. Docosahexaenoic acid (DHA) is an n-3 polyunsaturated fatty acid (PUFA) with a 22 carbon chain (C22:6) [6, 7]. DHA supplied in the cow diet modified the lipid composition of bovine oocytes [1]. DHA also increase membrane fluidity: its incorporation into the membrane facilitate membrane deformation [8]. Moreover, DHA supplementation in the culture medium (1 to 20 µM) increases the proliferation and steroidogenesis of granulosa cells [9] and increases oocyte quality *in vitro* and blastocyst rate on day 7 [7]. Furthermore, because lipids and especially n-3 PUFA are easily oxidised, compensatory antioxidant supplementation were also assessed *in vitro*. Supplementation with carnosine, a reactive oxygen species scavenger, into the *in vitro* development (IVD) medium accelerated the kinetics from the 4-cell stage to compaction of the morula, and it also increased the post-thaw viability [10].

We hypothesized that DHA increases the success rate of frozen embryo transfer by improving embryo quality as well as by reducing their cryosensitivity due to its incorporation into the membrane. Thus, we evaluated the effect of DHA and carnosine supplementation in *in vitro* maturation (IVM) and/or IVD media, in the absence of serum, on embryonic quality using both the cryosurvival rate and a marker of pluripotency (SOX2) of bovine expanded grade-1 blastocysts, after thawing.

2. Materials and Methods

No experiments with living animals were performed. All chemicals were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France), unless otherwise stated in the text.

2.1. In vitro embryo production

COCs were retrieved from bovine ovaries collected at a local slaughterhouse. Follicles from 2 to 8 mm in diameter were punctured with an 18G needle linked to a vacuum pump and added to a 50 mL Falcon tube containing 1 mL of flushing medium (Euroflush, IMV Technologies, L'Aigle, France) maintained at 37 °C.

Only COCs with at least three layers of compact cumulus cells, with homogenous and non-granular cytoplasm, were selected [11] and washed two times in Euroflush at 37 °C and then a third time in serum-free IVM medium before undergoing maturation for 22 h. The maturation medium comprised TCM-199, supplemented with 4g/L fatty acid free (FAF) bovine serum albumin (BSA), 10 ng/mL epidermal growth factor (EGF), 10 µg/mL porcine follicle-stimulating hormone (pFSH)/porcine luteinising hormone (pLH), 1 µg/mL 17β-oestradiol and 5 µg/mL gentamycin. All COCs were incubated at 38.5 °C for 22 h under a maximally humidity atmosphere with 5% CO_2.

IVF was performed as described previously [12]. The same semen from the same bull and the same ejaculate were used for all the experiments. Therefore, there is no male effect among the experiments. Eighteen hours after fertilisation, all presumptive zygotes were cleared of cumulus cells and of spermatozoa attached to the zona pellucida by centrifugation. Zygotes were washed twice in Euroflush then twice in IVD medium. Zygotes were cultured in a 30 µL micro-drop of IVD medium at 38.5 °C in a maximally humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 and covered with mineral oil (Liquid Paraffin, Origio, Måløv, Denmark). IVD_C medium consisted of a serum-free, synthetic oviductal fluid control (SOF_C) whose composition is described in [13], supplemented with 6 g/L of FAF BSA.

2.2. Embryo quality assessment

The zygote cleavage rate was assessed under a stereoscopic microscope at 20× magnification 48 h post-fertilisation (D2). The total developmental rate at D6 was evaluated by the numbers of morula/blastocysts. Additionally, the blastocyst developmental rate and embryo quality were recorded on D6–D8 (D0 = day of IVF), according to the International Embryo Technology Society (IETS) morphological criteria (Chapter 9 and Annex D, IETS Manual, 3rd edition). Only expanded grade-1 blastocysts were used for the quality assessment experiment – that is, expanded blastocysts with a compact inner cell mass, uniformly coloured blastomeres, few irregularities or excluded cells and an intact and smooth zona pellucida. After 7 days of development, the expanded grade-1 blastocysts were frozen. Those that were not completely expanded were frozen after 24h of additional culture, i.e. on day 8. To indicate the development of D8 expanded grade-1 blastocysts, the rate of expanded grade-1 blastocysts in Figure 2 and 3 include those frozen on D7 and those newly appeared on D8 since it was considered that an expanded blastocyst on D7 will still be present on D8.

2.3. Embryo Freezing and Thawing

Embryos were frozen slowly in ethylene glycol sucrose solution, following a protocol described previously [12]. Briefly, expanded grade-1 blastocysts were washed in embryo-holding medium (EHM, IMV Technologies, L'Aigle, France). Then, embryos were placed in 1.5 M ethylene glycol freezing medium (IMV Technologies) supplemented with 0.1 M sucrose for 10 min at room temperature. Embryos were mounted in groups of up to 9 embryos in 250 µL straws. The straws were placed in the cryochamber of the freezer (Freeze Control®, Cryologic, Melbourne, Australia), previously equilibrated at -6 °C. After 2 min at -6 °C, the seeding was induced manually. The temperature was lowered at a rate of 0.3 °C/min, down to -32 °C. When this temperature was reached, the straws were directly immersed in liquid nitrogen at -196 °C, before being stored.

For post-thawed experiments, straws containing the embryos were thawed for 3 s in ambient air and then immersed in a water bath at 35 °C for 30 s. Their contents were poured into a Petri dish, thus mixing the different phases. After 15 min at room temperature, the embryos were washed for 5 min

in EHM at room temperature. Finally, the embryos were washed and then cultured in SOF (Minitüb, Gmbh, Tiefenbach, Germany), supplemented with 2% of minimum essential media (MEM) and 1% of basal medium eagle (BME), 0.33 mg/mL sodium pyruvate, 1% oestrus cow serum and 6g/L FAF BSA, in the incubator under 5% CO_2, 5% O_2 at 38.5 °C for 48 h. The cryosurvival of the embryos was evaluated at 24 and 48 h post-thawing, by recording the re-expansion and hatching rates.

2.4. Immunostaining

The presence of FFAR4 (also known as GPR120), a receptor able to bind DHA, was determined by immunofluorescence on COCs, embryos at the 2-cell stage and expanded blastocysts, as described previously [14]. Briefly, COCs, 2-cell stage embryos and expanded blastocysts were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. Following 15 min of permeabilization in 0.5% Triton in PBS, oocytes and embryos were incubated in PBS with 10% FCS for 3 h and in PBS with 5% FCS and 2% BSA for 2 h. The FFAR4 antibody was produced in rabbits (homemade antibody). Samples were then incubated at 4 °C overnight with rabbit anti-FFAR4 (9.5 ng/mL) or rabbit IgG before immunisation for the negative control (same concentration), both diluted in PBS with 2% BSA. After four washes in PBS with 0.2% BSA, samples were incubated in the dark with secondary antibody, Alexa Fluor488–conjugated goat anti-rabbit IgG (Life Technologies, Saint Aubin, France) diluted 3:1000 for 3 h at ambient temperature. Another four washes in PBS with 0.2% BSA were performed, and the nuclei of the samples were counter-labelled with Hoechst 33342 (5 µg/mL) (Thermo Fisher Scientific, Illkirch-Graffenstaden, France) for 6 min at 37 °C.

After the cryosurvival evaluation the presence of SOX2 and CDX2 was determined by immunofluorescence. All surviving embryos were co-immunolabeled with SOX2 and CDX2 to assess the state of pluripotency (SOX2) as well as the proliferation of the trophectoderm (CDX2) [15]. Embryos were fixed in 4% paraformaldehyde in PBS for 15 min. The blastocysts were then washed twice in a solution of TCM199 HEPES, supplemented with 0.1% Triton and 2% FCS, for 5 min. Embryos were permeabilised in PBS, 0.5% Triton, 1% BSA and 10% FCS, for 1 h at 37 °C. Embryos were incubated with

two primary antibodies, goat polyclonal anti-SOX2 (AF2018, R&D Systems, Minneapolis, Minnesota, USA) and mouse monoclonal anti-CDX2 IgG (sc-166830, Santa Cruz Biotechnology, Dallas, Texas, USA), diluted 1/250 and 1/200, respectively, in PBS supplemented with 0.1% Triton and 1% BSA for 1 h at 37 °C. The embryos were then rinsed for 45 min in the rinsing solution at room temperature. Then, they were incubated with two polyclonal secondary antibodies, anti-goat IgG conjugated to indocarbocyanine Cy3 (Jackson Immunoresearch, West Grove, Pennsylvania, USA) and anti-mouse IgG conjugated to Alexa Fluor 488 (Abcam, Cambridge, United Kingdom), diluted 1:1500 in PBS containing 0.1% Triton and 1% BSA for 1 h at 37 °C. After 45 min of washing, the nuclei of the embryos were counter-labelled with Hoechst 33342 (5 µg/mL) (Thermo Fisher Scientific) for 6 min at 37 °C. After rinsing, the embryos were mounted on slides in mounting medium (Fluoromount-G™, Invitrogen™, Thermo Fisher Scientific, Illkirch-Graffenstaden, France).

2.5. Confocal Microscopy and Image Processing

A confocal laser scanning microscope (LSM700, Carl Zeiss Microscopy GmbH, Munich, Germany) was used to examine the immunofluorescence. This microscope is driven by the Zen black acquisition software (ZEISS). The acquisition parameters were as follows: objective 20×0.8; gain between 700 and 800 depending on the channel; laser power between 0.3% and 4.5%; excitation of Alexa Fluor 488 at 488 nm, Cy3 at 555 nm and Hoechst 33342 at 405 nm; optimal resolution of 2048×2048 pixels/image; scanner speed at 7 (scanning time 0.79 µs/pixel, or 23.23s/image); acquisition interval of 1.00 µm for z-images.

To acquire the images of FFAR4 immunofluorescence, ‘Snap’ using the ZEN black software (release 2009) was performed. Z-images of SOX2/CDX2 immunofluorescence were acquired with ‘Z-stack’ using the ZEN black software. This process compiles several images with varying focal planes to produce an image with a greater depth of field. To exploit and extract information concerning the number of cells, the following image treatments have been applied: ‘Maximum intensity projection’, which allows projecting the pixel with the highest value on the z-axis so that in a single two-dimensional image all

the dense structures of a given volume are observed, and 'Filter median', to reduce the noise of the image. Cell counting was then performed with Fiji software (Image J, release 2.3.0, National Institutes of Health, Bethesda, MD) using the 'Multi-point Tool', which allows manual counting.

2.6. Experimental Design

Experiment 1. *Evolution of the allocation of SOX2 within grade-1 expanded blastocysts between J7 and J9.*

In the following experiments (experiment 3 and 4), the grade-1 expanded blastocysts were selected on Day 7 or Day 8 and then frozen and then their cryosurvival was evaluated over 48h, before the SOX2 immunostaining was performed. To know the evolution of the allocation of SOX2 during the whole process, grade-1 expanded blastocysts were selected on Day 7, half was immunolabelled for SOX2, while the other half was cultured for 48h to mimic the cryosurvival period, before being immunolabelled for SOX2.

Experiment 2. *Analysis of the expression of* free fatty acid receptor-4 *(FFAR4) in COCs, 2-cell stage embryos and expanded blastocysts.*

DHA is a ligand of FFAR4 and exerts some of its physiological effects through this receptor. To confirm that DHA was able to act through FFAR4 during early embryo development, the presence of this receptor was determined on COCs, 2-cell stage embryos and expanded blastocysts using immunofluorescence.

Experiment 3. *The effects of adding DHA to the oocyte maturation medium on the development and quality of in vitro produced bovine embryos*.

To determine which DHA concentration is beneficial for embryo development, COC were matured in IVM medium supplemented with either 20 µM DHA (IVM_{DHA20}) or 100 µM DHA (IVM_{DHA100}). DHA has

been diluted in DMSO and, therefore, the controls also had the same DMSO level, either 0.02% or 0.1%. To avoid lipid oxidation, following the addition of DHA, 5 µg/mL carnosine was added. All oocytes were *in vitro* fertilised and cultured in serum-free medium (IVD_C). After 7 days of *in vitro* culture, all grade-1 blastocysts were frozen slowly. After thawing, the cryosurvival of embryos was evaluated by recording the re-expansion and hatching rates after *in vitro* culture for additional 48h. At the end of the cryosurvival evaluation, to assess the embryo quality, SOX2 immunostaining was performed on the viable embryos, i.e. expanded/hatched blastocysts. Six experimental groups were constituted corresponding to 11 independent experiments:

- IVM_C: without supplementation (n = 800 COCs)
- IVM_{DHA20}: IVMc + 20 µM DHA in 0.02% DMSO + 5 µg/mL carnosine (n = 773 COCs)
- IVM_{DHA100}: IVMc + 100 µM DHA in 0.1% DMSO + 5 µg/mL carnosine (n = 492 COCs)
- $IVM_{DMSO\ 0.02}$: IVMc + 0.02% DMSO (n = 297 COCs)
- $IVM_{DMSO\ 0.1}$: IVMc + 0.1% DMSO (n = 499 COCs)
- IVM_{CARN}: IVMc + 5 µg/mL carnosine (n = 458 COCs)

Experiment 4. *The effects of adding 20µM DHA to the oocyte maturation medium and/or to embryo development medium on the development and quality of bovine in vitro produced embryos.*

To assess the effects of DHA supplementation throughout embryo culture, COCs were matured in IVM medium in the absence (IVMC) or the presence of 20 µM DHA and 5 µg/mL carnosine (IVMDHA20) and in vitro cultured in IVD medium in the absence (IVDC) or the presence of 20 µM DHA and 5 µg/mL carnosine (IVDDHA20) until the freezing day (D7 or D8) (Figure 4). The period of culture is a period where embryo will undergo cell membrane production. Indeed, cells will proliferate from one to several hundred cells, suggesting a significant need for lipids involved in membrane composition. We hypothesized that the lipid requirement could increase the incorporation of DHA into the membranes and thus increase membrane fluidity.

Four experimental groups were formed:

- IVM_C / IVD_C (n = 463 COCs)
- IVM_C / IVD_{DHA20} (n = 475 COCs)
- IVM_{DHA20} / IVD_C (n = 482 COCs)
- IVM_{DHA20} / IVD_{DHA20} (n = 480 COCs)

After 7 or 8 days of *in vitro* culture, all grade-1 blastocysts were frozen slowly. After thawing, the cryosurvival of embryos was evaluated by reculturing embryos for 48 h and by recording the re-expansion and hatching rates. At the end of the cryosurvival evaluation, to assess the embryo quality, SOX2 immunostaining was performed, at D9 or D10 depending on the freezing day. To prevent DHA from interfering with sperm and impacting the fertilisation rate, the IVF medium has not been supplemented.

2.7. Statistical Analysis

Data analysis was performed by using SAS software (Statistical Analysis Software release 9.4, SAS, Cary, North Carolina, United states). For qualitative data, multivariate analyses were performed by using logistic regression mixed models with post hoc Tukey–Kramer correction (GLIMMIX procedure), including the fixed effect of the experimental group and the random effect of the embryo production session. For the cryosurvival and immunostaining data, the fixed effect of the freezing day was added to the model as well as its interaction with the experimental group. For quantitative data, the MIXED procedure was performed with post hoc Tukey–Kramer correction, the fixed effect of the experimental condition and the random effect of embryo production session. For the immunostaining data, the fixed effect of the freezing day was added to the model as well as its interaction with the experimental group. The differences were considered significant when p-adjusted < 0.05. The data are presented in the tables and figures as the adjusted least squares mean ± standard error of the mean are presented.

3. Results and Discussion

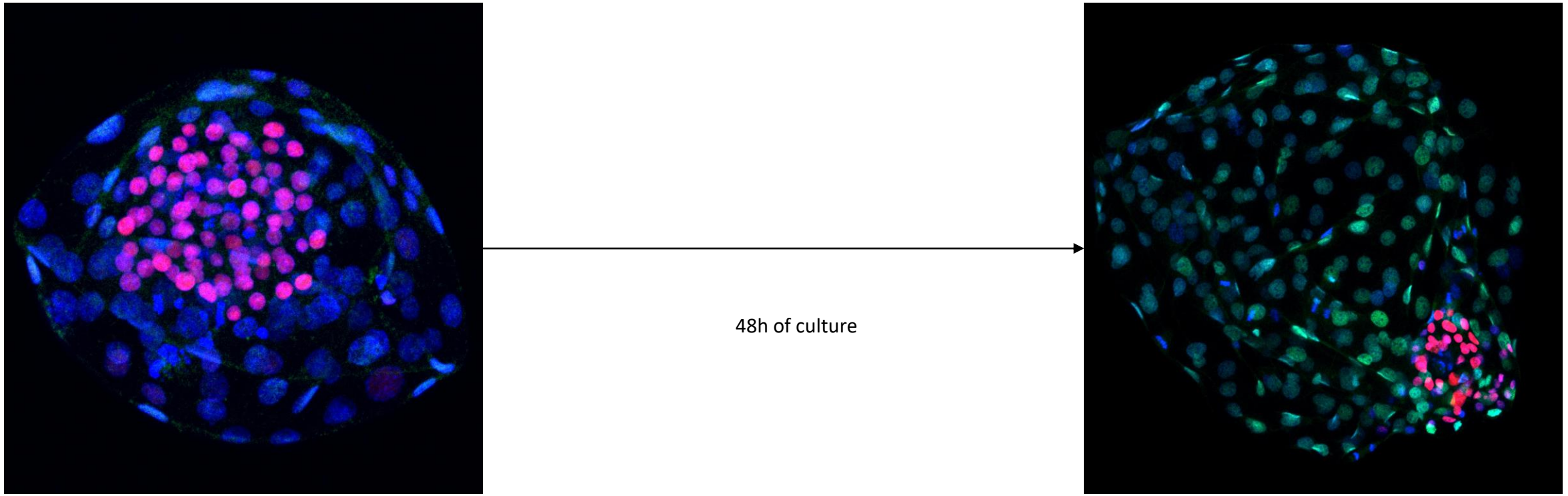
3.1. Evolution of the allocation of SOX2 within grade-1 expanded blastocysts between J7 and J9.

Between Day 7 and Day 9, the number of total cells was 1.8-fold increased. On Day 7, the grade-1 expanded blastocysts averaged 183.3 total cells versus 334.6 cells 48h later on Day 9 ($p < 0.0001$). Regarding the number of SOX2 cells, a decrease was observed between Day 7 and Day 9, respectively 76.4 cells vs. 50.9 cells, $p = 0.003$ (Figure 1). The increase in the number of total cells and the decrease in the number of SOX2 cells resulted in a decrease in the ratio of SOX2 cells / total cells. Indeed, SOX2 cells represented 41.6% of the grade-1 expanded blastocysts on Day 7 and represented only 15.1% of the blastocysts on Day 9. This preliminary experiment highlighted that between Day 7 and Day 9 a strong proliferation of the trophectoderm and a decrease in the number of SOX2 cells is expected.

Although SOX2, therefore the presence of pluripotent cells, is required in the embryo so that a gestation is possible, the decrease in the number of cells expressing SOX2 is expected and correspond to the differentiation of the first lineage within the inner cell mass [16].

The establishment of cell lines during pre-implantation development should be related to a decrease in the expression of some pluripotency factors because some of the cells engage in a developmentally restricted process (TE and the hypoblast). Thus, the decrease in the number of SOX2-positive cells is linked to its restriction to the primary epiblast [16]. In addition, in line with these results, researchers have shown that in mice, the expression of GATA-binding protein 6 (GATA6), which is a known marker of the hypoblast [17], seems to repress SOX2 expression [18]. The decrease in the number of SOX2 cells between Day 7 and Day 9 therefore seems physiological. The establishment of these tissues and their allocation during early stages of development in cattle is therefore a quality criterion [3, 19].

MIV_c / CIV_c	Number of BE Q1 at D7	BE with SOX2 positive cells	Total Cell Number	SOX2 positive Cell Number	SOX2 positive /Total Cell (%)
D7	19	100%	183.3 ± 16.9^a	$76,4 \pm 6.7^a$	$41,6 \pm 2.0^a$
D9	18	100%	334.6 ± 17.3^b	$50.9 \pm 6,8^b$	15.1 ± 1.0^b
p value of day effect			<0.0001	0.0028	<0.0001



BEQ1 on D7

BEQ1 on D9

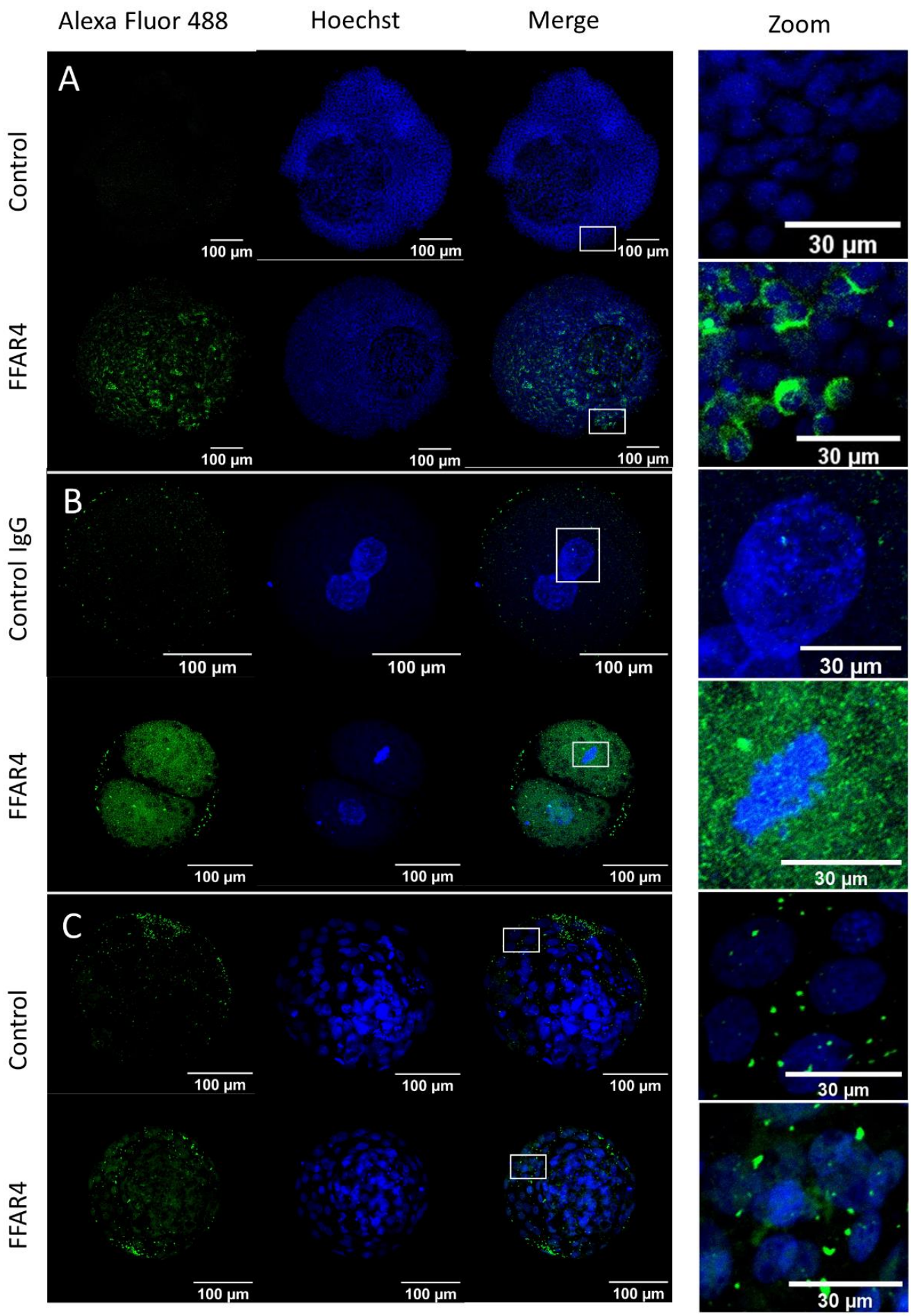
3.2. Free Fatty Acid Receptor 4 (FFAR4) Expression in Cumulus Oocyte Complexes (COCs), 2-Cell Stage Embryos and Expanded Blastocysts.

FFAR4 protein was detected by immunofluorescence in COCs, 2-cell stage embryos and expanded blastocysts by using a customized specific antibody against the bovine FFAR4 protein (**Figure 2**). The green fluorescence corresponding to FFAR4 was localised preferentially at the periphery of cumulus cells, and cytoplasmic but not nuclear staining was observed (**Figure 2*A***). Concerning the 2-cell stage embryos, the staining level was high and evenly distributed between the cytoplasm and nuclei (**Figure 2*B***). Regarding the expanded blastocysts, the immunostaining seemed to be localised similarly to the Hoechst labelling, which indicates that FFAR4 was localised in the nucleus, although there was some cytoplasmic staining (**Figure 2*C***).

We have reported the presence of FFAR4 not only in COCs, as previously described [14], but also in 2-cell embryos and D7 blastocysts. The presence of FFAR4, a membrane receptor able to bind DHA, suggests that DHA might exert an effect throughout oocyte maturation to the early steps of embryo development. It has been reported that FFAR4 is present on bovine oocytes and cumulus cells [14] and that its pharmacological activation by the agonist TUG891 would reproduce in part the effects of DHA supplementation, by increasing the developmental rate [14]. These results suggest that in addition to exerting a mechanical effect on membrane fluidity, DHA could also exert signalling effects up to the blastocyst stage. It is therefore interesting to assess the DHA effects not only during oocyte maturation but also during IVD of bovine embryos to assess its beneficial effect.

3.3. Effect of DHA During Oocyte Maturation on the Development and Quality of Bovine In vitro Produced Embryos.

The addition of 20 or 100 µM DHA in the IVM medium did not affect the developmental rate and the quality of expanded blastocysts at D7 and D8. The addition of 5 µg/mL carnosine or 0.02% or 0.1%


Alexa Fluor 488
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A
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Control IgG
FFAR4
C
Control
FFAR4
100 µm
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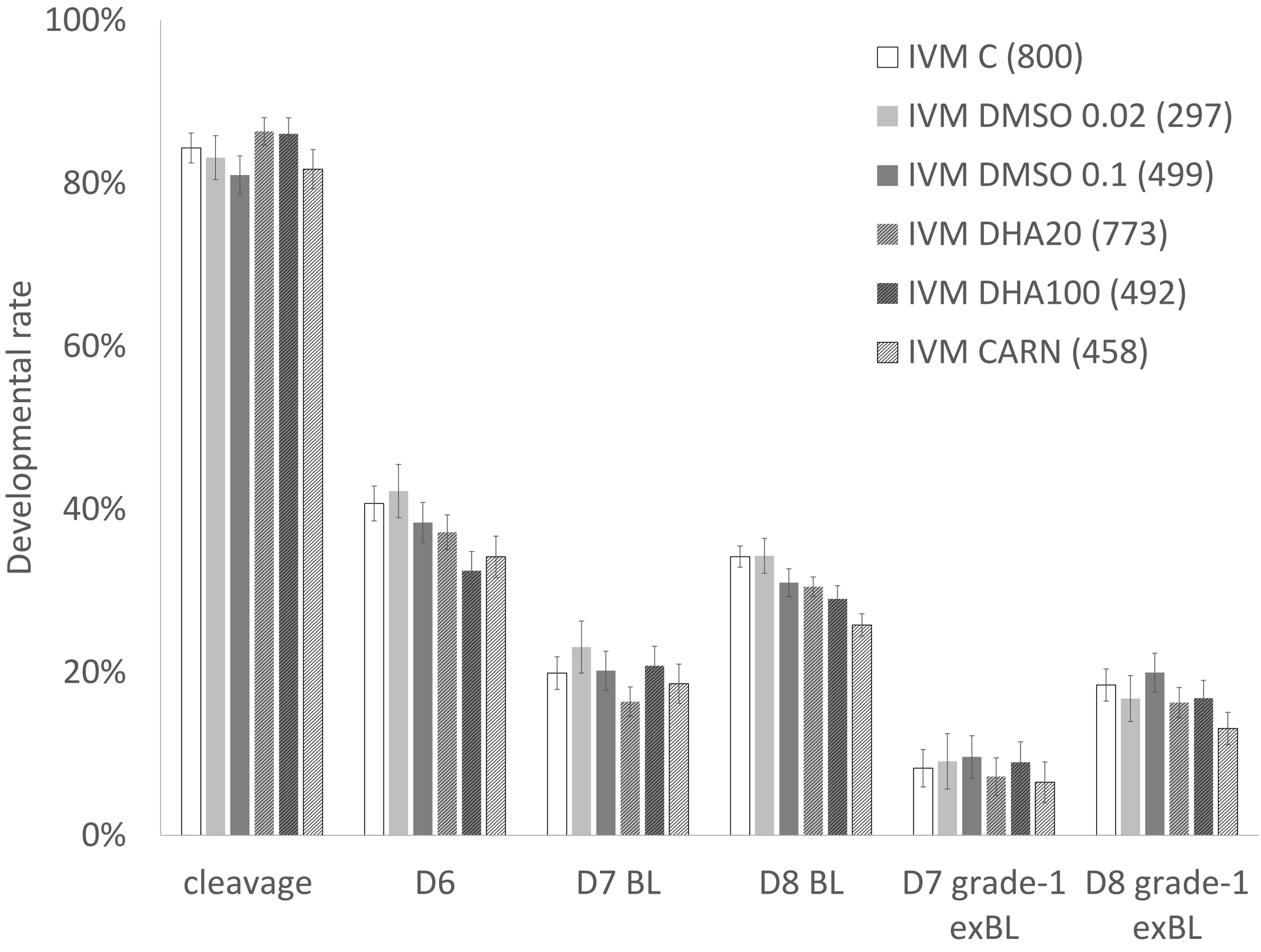
dimethyl sulphoxide (DMSO) in the maturation medium did not induce a change in the developmental rate compared with the control (**Figure 3**).

These results are partly in agreement with the literature [7, 20]. In fact, concerning the developmental rate, the addition of 12.5 or 25 µM DHA to the IVM of porcine oocytes did not alter the cleavage and blastocyst rates compared with the control [20]. When 1 or 10 µM DHA was added to the IVM medium of bovine oocytes, the cleavage and the blastocyst rate were increased. A higher DHA dose, 100 µM, decreased both the cleavage and blastocyst rates [7], contrary to the present study. These discrepancies among studies could be partly due to differences between species and in medium compositions such as the use of serum-free media in the present study, as serum is known to accelerate the development kinetics of *in vitro*–produced embryos [21]. In contrast to the literature, the antioxidant used in the present study, carnosine – which is physiologically present in bovine oviductal fluid after ovulation [22], – did not affect the developmental kinetics of embryos. It had been reported that embryos cultured *in vitro* with 5 µg/mL carnosine showed increased developmental kinetics, particularly between the first cleavage and the compaction of the morula [10]. In fact, the kinetic from 4-cell stage to compaction was shorter when embryos were cultured in the presence of carnosine compared with control (59.9 *vs* 51.8 hours). We therefore expected to observe an increased developmental rate at D6 (morulae + blastocysts). However, we did not observe an increase at D6 or after. Contrary to our study, carnosine was only added in the IVD medium containing serum; this could suggest that the carnosine effect depends on serum. It would be possible that carnosine and DHA exhibit antagonistic effects since the development rates were always lower when the maturation medium was supplemented with carnosine alone. The addition of an antioxidant to culture media, to counter the oxidative effect of long-chain fatty acids, is used frequently and allows maintaining antioxidant stability in culture system by reducing the intracellular levels of oxygen reactive species [23].


100%
80%
60%
40%
20%
0%
Developmental rate
IVM C (800)
IVM DMSO 0.02 (297)
IVM DMSO 0.1 (499)
IVM DHA20 (773)
IVM DHA100 (492)
IVM CARN (458)
cleavage
D6
D7 BL
D8 BL
D7 grade-1 exBL
D8 grade-1 exBL

After thawing, the re-expansion and hatching rates did not differ between groups **(Table 1)**. The presence of carnosine or DMSO at different concentrations did not affect the cryosurvival rate compared with the control.

Table 1. The re-expansion and hatching rates of expanded grade-1 blastocysts matured *in vitro* in the presence of 20 or 100 µM docosahexaenoic acid (DHA), after warming and 24 and 48 h of culture.

	Thawed embryos	Reexpansion rate		Hatched embryos
	n	After 24h	After 48h	After 48h
Groups (*p adjusted)*		0.338	0.219	0.304
IVM_{C}	49	91.9 ± 3.9	91.8 ± 3.9	69.4 ± 6.6
$IVM_{DMSO\ 0.02}$	32	95.3 ± 4.6	90.5 ± 6.4	66.7 ± 10.3
$IVM_{DMSO\ 0.1}$	21	93.7 ± 4.3	96.9 ± 3.1	87.5 ± 5.8
IVM_{DHA20}	52	84.8 ± 5.0	78.9 ± 5.7	69.2 ± 6.4
IVM_{DHA100}	32	77.9 ± 7.5	78.1 ± 7.3	59.4 ± 8.7
IVM_{CARN}	21	90.4 ± 6.5	85.7 ± 7.6	76.2 ± 9.3

Please see the Materials and Methods for full details of the groups. The data are presented as the least squares mean ± standard error of the mean. Data within the same column and experiment with different superscripts are statistically different (p-adjusted < 0.05).

There were no differences in the cryosurvival rate, indicating that 20 or 100 µM of DHA did not affect the cryosurvival of the expanded grade-1 blastocysts frozen slowly on D7. The addition of carnosine did not affect the cryosurvival rate, a finding in contrast to the literature. Indeed, authors reported that 5 µg/mL carnosine in the culture medium increased the cryosurvival rate 48 h after thawing compared with the control condition (70.4% *vs* 40.0%) [10]. It is possible that when combined with serum, carnosine would allow obtaining better quality embryos, more capable of withstanding the freezing process. Similar results were obtained when embryos were cultured in medium containing serum and in the presence of 0.5µM of resveratrol, a flavonoid antioxidant. Indeed, the authors demonstrated that the addition of resveratrol increased the cryosurvival rate (54.2% vs. 68.3%) [24].

The addition of DHA, DMSO or carnosine in IVM medium did not affect the proportion of embryos with SOX2-positive cells (p = 0.885). The number of cells and SOX2-positive cells did not differ between the

groups **(Table2)**. Only the percentage of SOX2-positive cells, i.e. the ratio of SOX2-positive cells to the number of total cells, differed without an impairment of the total cell number. By performing immunostaining on D9 embryos, we expected to obtain embryos with more cells and a reduced ratio of SOX2-positive cells to total cells (see above, Results and Discussion 3.1).

Table 2. The number of cells and SOX2-positive cells in expanded grade-1 blastocysts at day 9 (D9) of development, 48 h post-thawing.

			Number of cells		Mean ratio
	n	% Embryos with SOX2-positive cells	Total cells	SOX2-positive cells	SOX2-positive cell/total cells
Groups (*p adjusted)*		0.885	0.263	0.887	<0.0001
IVM_C	35	78.7 ± 9.2	143.1 ± 12.9	19.2 ± 3.4	13.7 ± 1.2 [a]
$IVM_{DMSO\ 0.02}$	27	81.5 ± 10.7	165.9 ± 15.9	17.1 ± 4.1	11.2 ± 1.1 [a,b]
$IVM_{DMSO\ 0.1}$	43	83.8 ± 7.7	162.4 ± 11.4	17.5 ± 3.0	11.0 ± 8.4 [b]
IVM_{DHA20}	23	86.7 ± 8.4	193.8 ± 15.4	16.5 ± 3.9	8.4 ± 0.8 [c]
IVM_{DHA100}	24	71.4 ± 12.5	157.6 ± 17.1	16.4 ± 4.4	10.4 ± 1.1 [b,c]
IVM_{CARN}	17	82.8 ± 13.0	169.5 ± 21.8	23.9 ± 5.5	13.3 ± 1.4 [a,b]

Please see the Materials and Methods for full details of the groups. The data are presented as the least squares mean ± standard error of the mean. Data within the same column and experiment with different superscripts are statistically different (p-adjusted < 0.05).

Regarding the cryosurvival rate, the addition of DHA, DMSO and carnosine in IVM medium did not show a significant effect on the number of cells and SOX2-positive cells in blastocysts after thawing. Even though an increase in the number of cells at D9 and a corresponding decrease in the number of SOX2-positive cells was expected, compared with D7 expanded grade-1 blastocysts (**Figure 1**), no difference in the number of cells were observed.

The addition of 20 µM DHA in the IVM medium appears to be beneficial to embryo development, allowing a reduction in the percentage of SOX2-positive cells without impairment in the total number of cells, probably at the origin of cell allocation of the first embryo cell lines.

DHA has already been reported to affect cellular functions through several mechanisms: transcription factor regulation, arachidonic acid production, membrane lipid composition modification or through

receptor binding [8]. Indeed, DHA is not only a component of membranes but it can activate receptors such as FFAR4 and peroxisome proliferator-activated receptor (PPAR, part of the β-oxidation pathway) [14, 25].

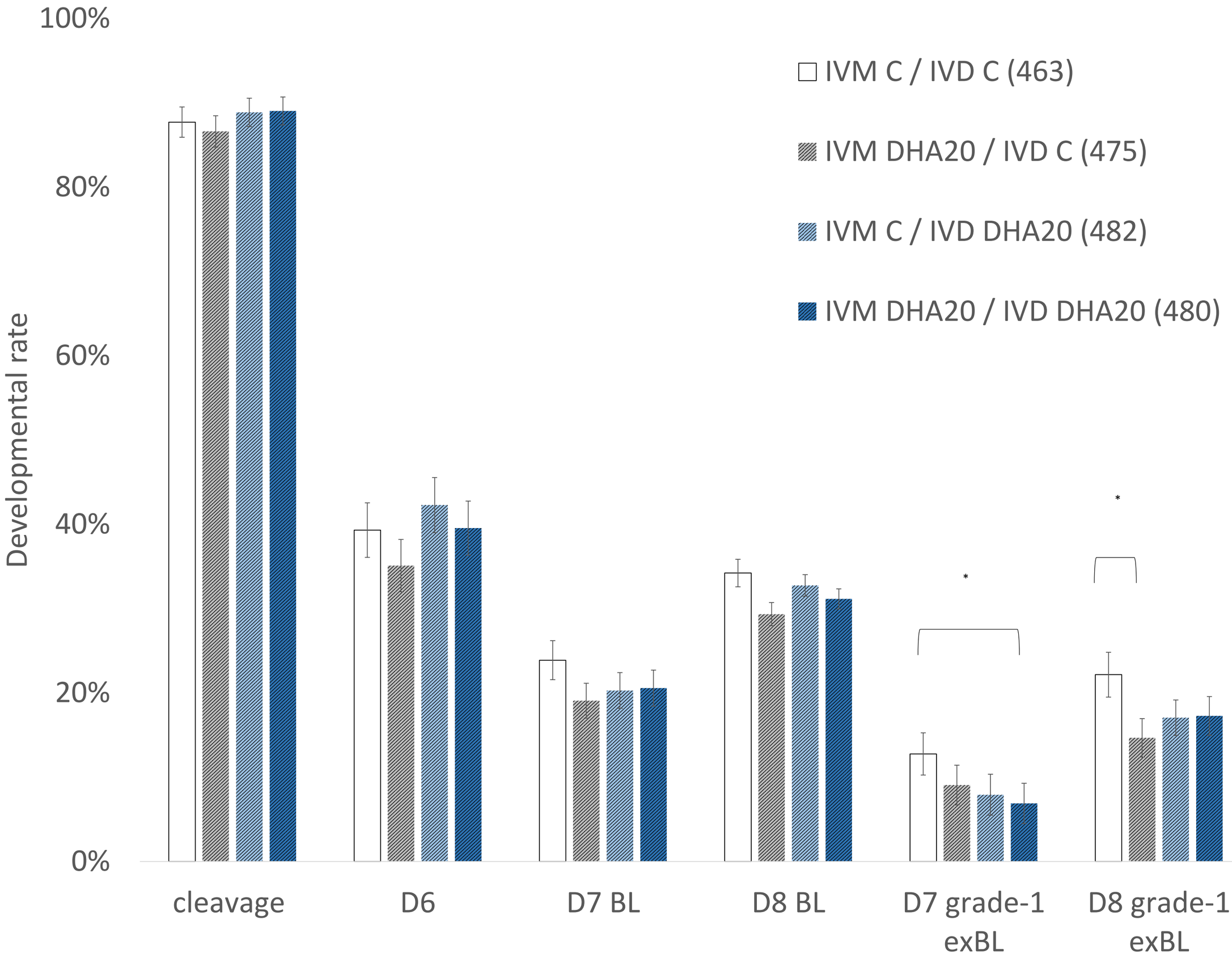
The lower percentage of SOX2-positive cells in DHA-treated embryos could be related to the higher number of cells and, therefore, to a DHA effect on cell proliferation. Indeed, 20 µM DHA stimulated cell proliferation and steroidogenesis in bovine granulosa cells [9], through FFAR4 and not PPAR for cell proliferation. TUG-891, a FFAR4 agonist, mirrored the effects of DHA on granulosa cell proliferation. Thus, FFAR4 could activate the extracellular signal-regulating kinase (ERK)1/2 pathway and activate the transcription factors linked to cell proliferation [14, 25].

Hence, DHA treatment leading to higher rate of SOX2-positive embryos could also suggest that when treated with DHA, embryos have a better transfer potential.

Considering all the results, we hypothesize that despite the absence of change in the developmental rate, the more favourable percentage of SOX2-positive cells observed in embryos treated with 20 µM DHA during IVM could result in a higher pregnancy rate. Future studies should thus focus on embryo transfer of such embryos to confirm this hypothesis.

3.4. Effect of Adding DHA to Oocyte Maturation Medium and/or to Embryo Culture Medium on the Development and Quality of Bovine In vitro–Produced Embryos

The addition of 20 µM DHA to oocyte maturation medium and/or to the embryo culture medium did not affect the cleavage rate at D2 or the developmental rate of D6 morulae and blastocysts as well as D7 and D8 blastocysts (**Figure 4**). However, 20 µM DHA affected embryonic quality at D7 and D8. Indeed, the addition of 20 µM DHA during the entire development (IVM + IVD) decreased the expanded grade-1 blastocyst rate at D7 compared with the control (6.9% ± 1.2% *vs* 12.8% ± 1.6%, *p*-adjusted = 0.041) (**Figure 4**). When added only in the IVM medium, it decreased the expanded grade-1 blastocyst rate at D8 compared with the control (14.7% ± 2.3% *vs* 22.2% ± 2.7%, *p*-adjusted = 0.045) (**Figure 4**). No difference was observed when 20 µM DHA was only added to the IVD medium.


100%
80%
60%
40%
20%
0%
Developmental rate
IVM C / IVD C (463)
IVM DHA20 / IVD C (475)
IVM C / IVD DHA20 (482)
IVM DHA20 / IVD DHA20 (480)
*
*
cleavage
D6
D7 BL
D8 BL
D7 grade-1 exBL
D8 grade-1 exBL

When added during the entire development (IVM + IVD), 20 µM DHA decreased the quality of the embryos, corresponding to a lower developmental rate of expanded grade-1 blastocysts at D7, without a change on D8, and therefore not explained by a developmental delay. When added during IVM only, 20 µM DHA reduced the embryo quality by decreasing the developmental rate of expanded grade-1 blastocysts on D8, without a change on D7. As these results differed from those obtained in the previous experiment (Figure 3) that was performed with more embryos, other experiments would be needed to demonstrate the decreasing effect of DHA on the blastocyst rate. Hence, the difference observed here could be due to the lower number of embryos per groups (475 vs. 773 COCs).

Even though DHA acts through several mechanisms, some of which are potentially beneficial to the oocyte or embryo, the concomitant activation of PPARγ by n-3 PUFA during IVM would induce lipid storage in lipid droplets of COCs and compromise the developmental competence of embryos [25, 26]. Thus, PPAR activation could potentially nullify other mechanisms that would potentially mediate the beneficial effects of DHA on embryos.

When DHA was added during IVD only, there was no effect on the quality of the embryos, which is in line with previous studies in which 1, 10 or 100 µM DHA added to the IVD medium of bovine embryos changed neither the cleavage rate nor the D8 blastocyst developmental rate [27]. It is therefore possible that the difference in the results comparing the DHA effects during IVM or IVD might be due to the presence of cumulus cells expressing PPAR. To confirm these hypotheses, it would be interesting to measure the expression of PPAR in expanded blastocysts and to assess the DHA effects while blocking PPAR activation.

After thawing, the re-expansion and hatching rates did not differ between the groups, indicating that 20 µM DHA in the oocyte maturation medium and/or the embryo culture medium did not affect the cryosurvival of the expanded grade-1 blastocysts at D9 or D10 depending on the freezing day. There was no effect of the freezing day and the experimental group on re-expansion and hatching rates (**Table 3**). However, there was a tendency for a decreased hatching rate in embryos frozen on D8

compared with embryos frozen on D7 (61.4% *vs* 71.5%, $p = 0.079$). A significant interaction between freezing day and groups was also reported on the re-expansion rate on D10 ($p = 0.045$), likely related to the fact that IVM_{DHA20} / IVD_{DHA20} corresponded to the lowest re-expansion rate on D7 embryos while it corresponded to the highest re-expansion rate on D8 embryos.

Table 3. The effects of adding 20 µM docosahexaenoic acid (DHA) to oocyte maturation medium and/or to the embryo culture medium on the cryosurvival rate of expanded grade-1 blastocysts, 24 and 48 h post-thawing.

	Freezing day	Thawed embryos	Re-expansion rate		Hatched embryos
		n	After 24h	After 48h	After 48h
p value freezing day			0.492	0.856	0.079
D7		161	88.0 ± 2.6	84.1 ± 3.7	71.5 ± 4.3
D8		153	85.3 ± 2.9	83.3 ± 3.9	61.4 ± 4.9
p value groups			0.799	0.626	0.244
IVM_C / IVD_C		85	89.4 ± 3.2	86.2 ± 4.1	74.5 ± 5.0
IVM_{DHA20} / IVD_C		65	84.0 ± 4.7	79.1 ± 5.9	63.2 ± 6.8
IVM_C / IVD_{DHA20}		77	86.2 ± 4.0	86.3 ± 4.4	68.0 ± 6.0
IVM_{DHA20} / IVD_{DHA20}		77	86.7 ± 3.9	82.4 ± 5.3	59.9 ± 6.3
p value freezing day x groups interactions			0.679	0.045	0.169
IVM_C / IVD_C	D7	53	90.6 ± 4.0	88.6 ± 4.7	79.4 ± 5.8
IVM_{DHA20} / IVD_C	D7	41	87.8 ± 5.1	85.5 ± 5.9	73.7 ± 7.2
IVM_C / IVD_{DHA20}	D7	36	88.9 ± 5.2	89.1 ± 5.4	75.9 ± 7.4
IVM_{DHA20} / IVD_{DHA20}	D7	31	83.9 ± 6.6	67.8 ± 9.2	53.8 ± 9.5
IVM_C / IVD_C	D8	42	88.1 ± 5.0	83.4 ± 6.2	68.9 ±7.5
IVM_{DHA20} / IVD_C	D8	24	79.2 ± 8.3	70.9 ± 10.0	51.3 ± 10.6
IVM_C / IVD_{DHA20}	D8	41	82.9 ± 5.9	83.0 ± 6.4	58.9 ± 8.2
IVM_{DHA20} / IVD_{DHA20}	D8	46	89.1 ± 4.6	91.3 ± 4.4	65.7 ± 7.4

Please see the Materials and Methods for full details of the groups. The data are presented as the least squares mean ± standard error of the mean. Data within the same columns and experiment with different superscripts are statistically different (p-adjusted < 0.05).

In the absence of serum, embryos exhibit slower development [2], which is why, in the present study, expanded grade-1 blastocysts were frozen on both D7 and D8. There was no difference in cryosurvival according to the group or the freezing day, except for a tendency for a lower hatching rate in embryos frozen on D8. This study confirmed that the expanded grade-1 blastocysts at D8 were of lower quality

[28, 29] or suggested that they could be more sensitive to the freezing/thawing process compared with morphologically similar embryos observed one day earlier (D7). These results are in line with the literature. Indeed, the best quality embryos (grade 1) would exhibit similar survival rates whether they were frozen on D7 or D8 [30]. Therefore, we did not expect a cryosurvival difference between D7 and D8 expanded grade-1 blastocysts. For this reason, we focused on the hatching rate, which provides insights on further development, because without hatching, pregnancy is impossible. Taken together, these data confirmed that D7 or D8 grade-1 blastocysts would survive similarly [30]. DHA treatment had no effect on cryosurvival when used during IVM, IVD or both.

Neither the freezing day nor the experimental group affected the proportion of SOX2-positive embryos (experimental group effect $p = 0.322$, freezing day effect $p = 0.597$) (**Table 4**). While the experimental group did not affect the number of cells and SOX2-positive cells ($p = 0.238$ and $p = 0.358$, respectively), the freezing day has a significant effect (**Table 4**). In fact, D7 embryos exhibited more cells 48 h after thawing compared with D8 embryos (194.4 ± 9.0 *vs* 166.4 ± 10.0, $p = 0.039$). Similarly, D7 embryos had more SOX2-positive cells 48 h after thawing compared with D8 embryos (26.5 ± 2.8 *vs* 16.2 ± 3.0, $p = 0.006$). The percentage of SOX2-positive cells was affected by both the freezing day and the experimental group. D7 embryos exhibited a higher SOX2-positive cell percentage compared with D8 embryos (13.06% ± 1.27% *vs* 9.55% ± 1.0%, $p < 0.0001$). Regarding the experimental group effect, the percentage of SOX2-positive cells was reduced in the IVM_C / IVD_{DhA20} group compared with the control (8.4% ± 0.9% *vs* 10.9% ± 1.1%, $p < 0.0001$). When DHA was supplemented during IVM or the entire culture process (IVM + IVD), the percentage of SOX2-positive cells was higher compared with the control group (13.1% ± 1.4%, $p = 0.002$, and 13.3% ± 1.4%, $p = 0.0004$, respectively) (**Table 4**). There was a significant interaction between the freezing day and the experimental group effect for the SOX2-positive cell percentage, meaning that the results could vary among experimental groups depending on the freezing day. This significant interaction means that depending on the experimental batch or the freezing day, the results do not vary in the same way. The post-thaw culture may be suboptimal and not suitable for the development of more demanding epiblast cells than trophectoderm cells [31-

33]. Indeed it would seem that the addition of 10% serum and 0.5% of glucose is beneficial for the proper development of hatched embryos [33] whose glycolytic activity and glucose uptake increase [34, 35]. The addition of DHA into the IVD medium of these embryos could be a way to protect ICM cells from the freezing/thawing process.

Table 4. The number of cells and SOX2-positive cells in expanded grade-1 blastocysts at day 9 (D9) or D10 of development, depending on the freezing day (D7 or D8), 48 h post-thawing.

				Number of cells		mean ratio
	n	Freezing day	% SOX2-positive Embryos	Total cells	SOX2-positive cells	SOX2/Total cells
Freezing day (*p adjusted)*			0.597	0.039	0.006	< 0.0001
D7	111		66.1 ± 7.4	194.4 ± 9.0 [a]	26.5 ± 2.8 [a]	13.06 ± 1.27 [a]
D8	92		62.1 ± 8.1	166.4 ± 10.0 [b]	16.2 ± 3.0 [b]	9.55 ± 1.0 [b]
Groups (*p adjusted)*			0.322	0.238	0.358	< 0.0001
IVM_C / IVD_C	66		63.3 ± 8.4	191.6 ± 10.6	20.6 ± 3.2	10.9 ± 1.1 [b]
IVM_{DHA20} / IVD_C	46		59.7 ± 10.3	169.0 ± 15.7	23.2 ± 4.5	13.1 ± 1.4 [a]
IVM_C / IVD_{DHA20}	54		75.3 ± 7.6	196.7 ± 10.9	16.6 ± 3.3	8.4 ± 0.9 [c]
IVM_{DHA20} / IVD_{DHA20}	37		56.7 ± 10.7	164.4 ± 15.7	25.0 ± 4.5	13.3 ± 1.4 [a]
Freezing day x Groups interactions (*p adjusted)*			0.193	0.157	0.134	< 0.0001
IVM_C / IVD_C	38	D7	62.8 ± 10.0	191.2 ± 13.2	23.3 ± 3.9	12.0 ± 1.2 [b]
IVM_{DHA20} / IVD_C	32	D7	74.8 ± 9.2	197.8 ± 14.0	25.9 ± 4.0	12.0 ± 1.2 [b]
IVM_C / IVD_{DHA20}	27	D7	68.0 ± 10.6	194.2 ± 16.2	18.4 ± 4.6	9.6 ± 1.1 [c]
IVM_{DHA20} / IVD_{DHA20}	14	D7	57.5 ± 15.0	194.7 ± 26.0	38.4 ± 7.2	20.5 ± 2.1 [a]
IVM_C / IVD_C	28	D8	63.8 ± 10.9	192.0 ± 16.7	18.0 ± 4.7	9.3 ± 1.0 [c,d]
IVM_{DHA20} / IVD_C	14	D8	42.5 ± 14.9	140.2 ± 28.1	20.4 ± 7.7	14.2 ± 1.8 [b]
IVM_C / IVD_{DHA20}	27	D8	81.4 ± 8.6	199.2 ± 14.7	14.8 ± 4.2	7.4 ± 0.8 [d]
IVM_{DHA20} / IVD_{DHA20}	23	D8	55.8 ± 12.7	134.1 ± 17.8	11.6 ± 5.0	8.4 ± 1.0 [d]

Please see the Materials and Methods for full details of the groups. The data are presented as the least squares mean ± standard error of the mean. Data within the same columns and experiment with different superscripts are statistically different (*p*-adjusted < 0.05).

The freezing day affected the quality of embryos. Indeed, embryos frozen on D7 contained more cells, suggesting a higher quality embryo, alongside more SOX2-positive cells. In this case, the reduced SOX2-positive cell number and percentage reported in D8 embryos was associated with a reduced number of cells, despite being one day older, and were therefore not indicative of better quality. These D8

454 embryos could be of lower quality and could have more difficulties facing the freezing protocol
455 compared with D7 embryos. Fewer cells could be the consequence of the destruction of some of the
456 cells both in the trophectoderm and ICM [31].
457 Considering both D7 and D8 embryos, although the number of total cells and SOX2 cells were not
458 different between the control and the group with DHA supplementation during only IVD, respectively
459 (20.6 vs. 16.6 cells and 191.6 vs. 196.7 cells), the percentage of SOX2-positive cells was significantly
460 decreased, suggesting accelerated development [15, 36]. On the contrary, when embryos were
461 supplemented with DHA during IVM or during IVM + IVD, embryos exhibited fewer cells and an
462 increased percentage of SOX2-positive cells, suggesting slower development. SOX2 overexpression
463 after D9 could reflect a suboptimal embryo development [36]. Therefore, DHA supplementation during
464 the entire culture process (IVM + IVD) could be detrimental to proper development. An embryo
465 without SOX2 expression would not be able to lead to a gestation, and future studies should focus on
466 a transfer study to assess this issue. Even though the developmental rates are similar, DHA could
467 increase the percentage of embryos expressing SOX2 and, consequently, the percentage of embryos
468 that would lead to gestation. Failure of epiblast development has been described *in vitro* and in vivo,
469 and several authors reported that between 23% and 65% of *in vitro* produced and transferred embryos
470 no longer had an embryonic disc, as reviewed by Ramos-Ibeas et al. [37]. In the present study,
471 regardless of the freezing day, 27.4% to 43.3% of the embryos lacked SOX2 cells and therefore epiblast,
472 after thawing and 48h of culture (Table 4).
473

474 *3.5. Limitations of the study*

475 We assessed the effect of DHA supplementation on *in vitro* oocyte and embryo quality. Nevertheless,
476 DHA was combined with both DMSO and carnosine, an antioxidant to limit the oxidation of DHA and
477 potentiate its effects, and thus we have reported the effect of this combination in the present study.
478 DMSO is regularly used in media for the production and the cryopreservation of bovine embryos [9,
479 14, 38, 39]. Due to its cellular toxicity and its impact on the maintenance of pluripotency [40], DMSO

should only be used at a low concentration [41], which was the case in the present study (0.02 or 0.1%). As mentioned before, the real embryo quality assessment is performed through embryo transfer studies. It would have been interesting to carry out these transfers to verify whether the improvement suggested by SOX2 positive cells staining in the present study would lead to an improvement in the pregnancy rates. Moreover, the numbers of embryos, especially in the last experiment were too low to enable to observe significant difference when comparing SOX2 positive embryo % in the different conditions. The major difficulty is to have enough statistical power to highlight a 12% difference (63% vs. 75%), requiring at least 130 embryos per experimental group. Nevertheless, It is important to further study the addition of DHA in IVM-IVD medium to assess whether it could enhance the % of SOX2 positive embryos (embryos containing pluripotent cells) and, therefore, the pregnancy rate.

4. Conclusion

In this study we used SOX2 immunostaining to study embryonic quality after exposure of oocytes and embryos to DHA, during IVM and/or IVD. SOX2-positive cell evaluation of grade-1 expanded blastocysts suggested that DHA might improve the embryo quality as well as the percentage of embryos that express SOX2, and therefore the embryos able to achieve a gestation. Future studies should confirm these results by carrying out embryo transfers to assess the beneficial effects of DHA supplementation.

Acknowledgments : This research was funded by ALLICE and APIS-GENE under the project CRYOPTIM.

Author Contributions: P.S., D.L.B., L.S. and S.E. conceived the experiments, S.J.I., V.S.F., DLB and L.L.B. conducted the experiments. S.J.I. analysed the results. S.J.I., V.S.F. and S.E. wrote the manuscript. S.J.I., V.S.F., S.E., P.S., D.L.B., L.S., L.L.B., A.D., T.J. and S.B. edited the manuscript. All authors have read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no competing interests.

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Figure Legends

Figure 1. Evolution of the allocation of SOX2 within grade 1 blastocysts between J7 and J9. SOX2 was immunolabelled in pink, CDX2 in green and all nuclei in blue.

Figure 2. Intracellular localisation of the free fatty acid receptor-4 (FFAR4) protein based on immunofluorescence of (A) a cumulus oocyte complex, (B) a 2-cell embryo and (C) an expanded blastocyst. Rabbit IgG was used as the negative control (Control IgG) for each developmental stage. Hoechst staining (in blue) indicates nuclei.

Figure 3. The developmental rate of bovine oocytes treated with 20 or 100 µM docosahexaenoic acid (DHA). The cleavage rate was checked on day 2 (D2) and the developmental rate on D6 (morulae + blastocysts [BL]), D7 (BL and expanded grade-1 blastocysts [exBL]) and D8 (BL and exBL). The developmental rate is presented as the least squares mean ± standard error of the mean of 11 independent experiments. The total number of cumulus oocyte complexes used per condition is noted in the figure key. Please see the Materials and Methods for full details of the groups. Differences were considered significant when p-adjusted < 0.05.

Figure 4. The effects of adding docosahexaenoic acid (DHA) to the oocyte maturation medium and/or to the embryo culture medium on the developmental rate. The cleavage rate was checked at day 2 (D2), and the developmental rate was checked at D6 (morulae + blastocysts [BL]), D7 (BL as well as expanded grade-1 blastocysts [exBL]) and D8 (BL as well as exBL). The developmental rate is presented as the least squares mean ± standard error of the mean of 11 independent experiments. The number of COCs used is been noted in the figure legend. Please see the Materials and Methods for full details of the groups. Differences were considered significant at p-adjusted < 0.05.